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# Improvement of Antibacterial Activity for Ceftazidime by Partially Purified Tannase from *Penicillium expansum*

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***Abstract*—**Tannase has wide applications in food, beverage, brewing, cosmetics and chemical industries and one of the major applications of tannase is the production of gallic acid. Gallic acid is used for manufacturing of trimethoprim. In the present study, a local fungal strain of *Penicillium expansum* $A_4$ isolated from spoilt apple samples gave the highest production level of tannase. Tannase was partially purified with a recovery yield of 92.52% and 6.32 fold of purification by precipitation using ammonium sulfate at 50% saturation. Tannase led to increased antimicrobial activity of ceftazidime against *Pseudomonas aeruginosa* and *S. aureus* and had a synergism effect at low concentrations of ceftazidime, and thus, tannase may be a useful adjuvant agent for the treatment of many bacterial infections in combination with ceftazidime.

***Keywords*—**Ceftazidime, *Penicillium expansum*, tannase, antimicrobial activity.

## I. Introduction

*PENICILLIUM* spp. is the major source of fruit decay in stored apples [1]. This fungus causes post-harvest decay of some fruits and has a worldwide distribution [2]. Also, it is the most virulent and economically significant pathogen, since it leads during the storage period to the spoiling of apples and the production of mycotoxins that have harmful effects of human health [1], [3]. Punctures, bruises or any physical damage on the fruit that occur during harvest and postharvest handling are the main avenues for the pathogen to infect apple fruit [3]. It is a psychrophilic blue mold and ubiquitous throughout the soil [4]. This fungus can grow best in cold and wet places [5]. It has also been found that the secretion of organic acids by *Penicillium expansum* not only leads to an increase the acidity of fruits, but also increased *Penicillium expansum* development, meaning an acidic environment could enhance *Penicillium expansum* development [6]. Despite the multiple and different hosts for this fungus, the symptoms are similar. In apples, the colors of the lesions may vary slightly with the variety of apples, from lighter-brown on green and yellow varieties to dark brown on the deeper red and other darker color varieties [4]. Different kinds of fruits were susceptible to *Penicillium expansum* such as McIntosh and Golden Supreme [7], [8].

Tannin material is a secondary metabolite that has the ability to dissolve in water. The tannin is found in many plants like cinnamon, pine, oak tree pile, eucalyptus, peels of fruits such as bannana and pomegranate, date tree and apple fruits besides to coffee and grape seeds [9]. The plant parts rich with tannin include: fruits, leaves, branches and bark [10]. Vegetable tannin extracts contain a variety of amorphous materials including polyphenolic tannins of large relative molecular mass, such as hydrolysable gallotannin and tannic acid, as well as a less-complex of non-tannins, such as flavones and gums [11]. Tannin is used in preparation of gallic acid (used in preparation of trimethoprim, dyes and pyrogallol), instant tea, Acron wine, coffee flavored soft drinks, clarification of fruit juice and beer and cleaning of highly polluting tannin from the effluent of the leather industry [12].

Tannase enzyme [tannin-acyl-hydrolase, E.C: 3.1.1.20] hydrolyze the ester and depside bonds in tannic acid to gallic acid and glucose. Although tannase is present in plants, animals and microorganisms, it is mainly produced by microorganisms like fungi, bacteria and yeast. Nowadays, the enzyme has wide applications in food, beverage, brewing, cosmetics and chemical industries. Tannase was used as clarifying agent overcome the problem of undesirable turbidity and bitterness. The biological treatment by tannase for tannin containing waste water is considered a better alternative [13]. This enzyme is used for preparation of instant tea, treatment of tannery effluents and tannin containing animal feed [14]. The gallic acid prodced by tannase is used in the manufacturing of broad-spectrum antibiotics, trimethoprim and synthesis of propyl gallate that is used in fats and oils as anti-oxidants [15]. The purpose of this research was to screen tannase production by *Penicillium expansum,* purification of tannase and detection of the antibacterial activity for combination of tannase with ceftazidime.

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## II. Materials and Methods

### A. Collection of Samples

The study included samples of a total of 20 spoilt apples collected at local markets in Baghdad City. These samples were cut into small cubes and placed on the surface of PDA (potato dextrose agar) using an L- shaped glass rod and incubated at 28 ºC for 5-7 days.

### B. Isolation and Characterization of Penicillium expansum

The fungal isolates formed were subcultured to purity, and fungal identification was carried out based on the colony morphologies and structural characteristics as observed under the light microscopy. The fungal characteristics were described and identified based on the description given by [16].

### C. Screening for Tanninolytic Activity on Pomegranate Pith Agar Medium

All fungal isolates were evaluated for their ability to produce tannase by culturing these isolates in the center of the PPA plate which contained per liter: pomegranate pith, 50 g; $NaNO_3$, 3 g; KCl, 0.5 g; $MgSO_4.7H_2O$: 0.5 g, $FeSO_4.7H_2O$: 0.01 g, $KH_2PO_4$, 1 g and agar-agar 30 g [17], then incubated at 28 °C for 5-7 days and then measured the clear zones formed due to the hydrolysis of tannin around the colony, the ratio of clear diameter to colony diameter was obtained, and this represented a semi-quantitative assay of tannase.

### D. Quantitative Analysis

Pomegranate pith medium without addition of agar was inoculated with three 0.5 cm diameter agar plugs of each selected fungal isolates and incubated in a rotary shaker at 150 rpm at 28°C for 5 days. Then, the culture was filtered using Whatman no. 5 filter paper and the filtrate was centrifuged at 5000 rpm for 10 min. The clear supernatant was used as the crude extracellular enzyme source and screened for the presence of tannase activity in the culture [10].

### E. Tannase Assay

Tannase assay was done according to the method that described by [18]. An aliquot of 0.5 ml of crude enzyme was added to 2 ml of substrate solution (0.35% of tannin dissolved in citrate buffer, 0.05 M), incubated at 30 ºC for 10 min then the reaction mixture was stopped by adding 2 ml of ethanol 95%. The reaction mixture was centrifuged by cooling centrifuge at 10000 rpm for 10 min. The optical density of supernatant was measured at 310 nm and the activity of tannase was estimated depending on the standard curve of tannin.

$$\text{Activity of tannase} = \text{O.D.} / \text{Slope}$$

### F. Estimation of Protein Content

The protein content of tannase was determined using Bradford dye method with BSA as a standard [19].

### G. Partial Purification of Tannase with Ammonium Sulfate Precipitation

The enzyme was precipitated with gradient concentrations (% saturation) of ammonium sulfate (0-40%, 40-50%, 50-60% and 70-80%). At each step, the precipitate was dissolved in small amount of optimum buffer and enzyme activity and protein concentration were circulated to measure the specific activity, and also the volume was measured.

### H. Effect of Tannase on Antibiotic Activity

The ceftazidime antibiotic was screened for antimicrobial activity using the macro dilution method [20] against *Pseudomonas aeruginosa* and *S. aureus*. Serial two-fold dilutions for ceftazidime were done by dissolving of ceftazidime in distilled water to give a stock concentration of 5000 µg/ml. The stock concentration was filtered using a 0.22 µm millipore filter. Two-fold serial dilutions of ceftazidime were made with distilled water to give concentrations ranging from 16 µg/ml to 128 µg/ml. A 0.1 ml of $1.5x10^8$ cfu/ml bacterial suspension (*P. aeruginosa* and *S. aureus* and, separately) was spread on the surface of Mueller-Hinton agar plates, left to dry for 15 min at room temperature. About 50µl from each dilution was placed in wells (7 mm in diameter) on Mueller-Hinton agar plates. On the other hand, 1 antibiotic (for each concentration): 1 tannase (vol/vol) and 1 antibiotic (for each concentration): 2 tannase (vol/vol) was mixed separately and 50µl from the mixture was placed in wells on Mueller-Hinton agar plates. The plates were then incubated at 37 °C for 18-24 h. The diameter of an inhibition zone for the mixtures and antibiotic alone were measured.

## III. Results and Discussion

### A. Isolation of Penicillium expansum

Eight isolates of *Penicillium expansum* (40%) were obtained out of 20 spoilt apple samples (Fig. 1) and identified to the likely species level based on the colony morphologies and structural characteristics. The colors of the lesions of the infected apples may vary with fruit variety, since it changed from lighter brown on green and yellow varieties to dark brown on the deep red varieties [4]. The most susceptible varieties of apple to *Penicillium expansum* infection are McIntosh, Golden Supreme and Golden delicious [7], [8]. With *Penicillium expansum* infections, an initial infected spots appears post-harvest, while fruit is in storage, and a sharp visible contrast can be seen between diseased and healthy tissue. Often, decaying tissue can be easily "scooped" out of the surrounding healthy tissue [7]. *Penicillium expansum* was found to grow most efficiently in the range of temperatures between 15°C and 27°C [14], while at temperatures lower and higher than these values, the growth was much slower. The growth rate of *P. expansum* was fastest at 90% relative humidity [20]. Blue mold caused by *Penicillium expansum* is a major post-harvest disease of apples [21]. The study reported by [1] found that *Penicillium expansum* and *P. griseofulvum* are the principal fungal species isolated from stored apples in Brazil.

### B.Screening of Tannase Producing by Penicillium expansum

All *Penicillium expansum* isolates were subjected to rapid screening for extracellular tannase production using pomegranate pith agar plates. Six *Penicillium expansum*

isolates were found to be positive for tannase activity. Among these active isolates, *Penicillium expansum* $A_4$ produced a higher clearing zone in comparison with the other isolates (Fig. 2). Nevertheless, to ensure that the best tannase producer was selected, all the positive isolates were subjected to secondary screening for tannase production in pomegranate pith liquid culture medium, and the results revealed that the productivity reached to 18.65 U/ml.

Depending on the culture conditions and type of strain, the tannase enzyme can be constitutive or induced showed different levels of production. Phenolic materials like gallic acid, tannic acid, pyrogallol and methyl gallate are considered as tannase inducers [22]. Pomegranate peels are considered an alternative useful source because it is regarded as a cheap substrate, widely available and for large-scale fermentation it serves as a feedstock; in contrast, pure tannic acid has a very high cost, is found in limited quantities and is unsuitable for the commercial production of the enzyme in large-scales [14]. In *Trichoderma harzianum* MTCC 10841, the pomegranate rind gave maximum productivity of tannase compared to jamun bark and amaltas leaves, and in the presence of Malt extract (2%) with $NH_4Cl$ (0.2%) [14]. A qualitative and quantitative analysis using tannic acid in agar medium and broth, respectively, was performed for detection of tannase production by *Aspergillus* spp and *Penicillim* spp, and it was found that most of these isolates have an ability to produce the tannase enzyme [23].

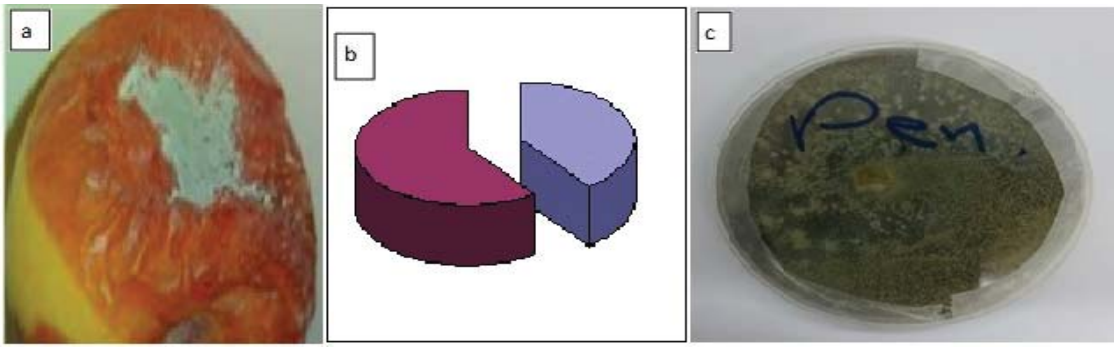



Fig. 1 (a) Apple sample infected with *Penicillium expansum*, (b) Isolation percentage of *Penicillium expansum* from spoilt apples samples, (c) *Penicillium expansum* grown in potato dextrose agar

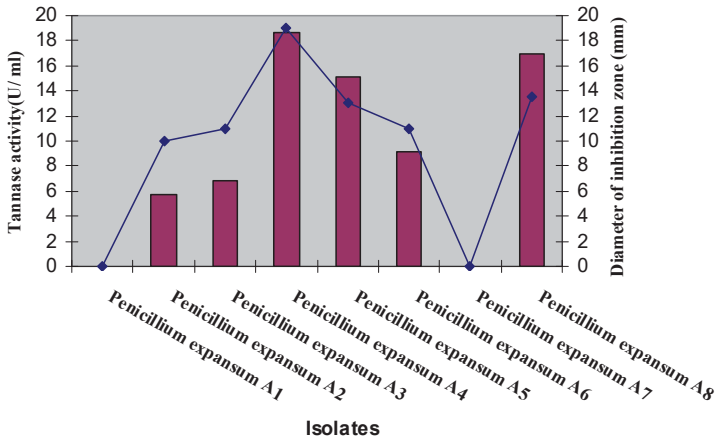



Fig. 2 Diameter of inhibition zone and tannase activity for *Penicillium expansum* isolates

*C. Partial Purification of Tannase*

An extracellular tannase was partially purified from the culture filtrate of *Penicillium expansum* grown in pomegranate pith medium (Fig. 3). The ammonium sulfate ranging 0-80 % is used for tannase precipitation. In the present study, 50% ammonium sulfate led to precipitate of tannase with higher specific activity, since the specific activity increased after fractionation with ammonium sulfate from 1.83 U/mg associated with crude tannase to 4.15 U/mg with respect to partially purified tannase and the summary of partial purification of tannase is presented in Table I. Tannase obtained from the fermentation process, without purification was used for commercial application; however, purification would enhance the extent of its efficacy as a clarifying agent and help in overcoming the problem of undesirable turbidity in beverages such as wine and beer which poses a quality problem [24]. Ammonium sulfate precipitation was followed by a dialysis step to remove the ammonium sulfate from the sample. To increase the specific activity of the enzymatic preparation, tannase should be concentrated. For that, classical methods such as salt or solvent precipitation, ultra filtration followed by ion exchange or size exclusion chromatography, as well as

solvent extraction are used [25]. Various techniques such as salt precipitation [1], solvent precipitation [26], and ultrafiltration [27], etc., were used in general for partially purified proteins, and in particular, for tannase purification.

TABLE I
STEPS OF PARTIAL PURIFICATION OF TANNASE FROM *PENICILLIUM EXPANSUM*

| Purification step | Size (l) | Tannase activity (U/ml) | Protein Conc.(mg/ml) | Specific activity(U/ml) | Total activity | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|---|
| Crude extract | 150 | 18.65 | 66.6 | 0.28 | 2797 | 1 | 100 |
| $(NH_4)_2SO_4$ precipitation | 60 | 43.14 | 24.3 | 1.77 | 2588 | 6.32 | 92.52 |

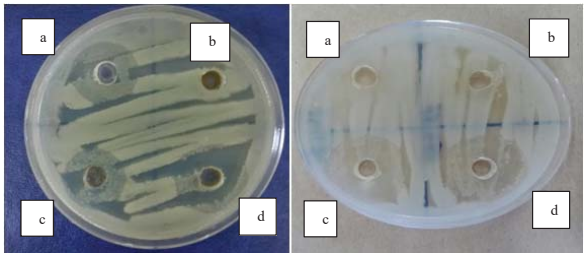
*D. Effect of Tannase on Antibiotic Activity*

This study showed that the ceftazidime at a concentration 128 µg/ml gave higher inhibition zone (31 mm) against *Pseudomonas aeruginosa,* while the concentration 16 µg/ml showed lower inhibition zone (24 mm) (Table II). In the case of ceftazidime combination at different concentrations with the purified tannase at 1:1 (vol/vol), it was found that in the concentrations 16 µg/ml and 32 µg/ml that the diameter of inhibition zones was increased to 25 mm and 27 mm, respectively. From this result, we can see that the combination of tannase with the low concentrations of ceftazidime led to an increase the antibiotic activity against this bacterium, but in the higher concentrations of ceftazidime, the tannase did not lead to any increase in antibacterial activity of ceftazidime. In the case of using 1 antibiotic (for each concentration): 2 tannase (vol/vol), the antibacterial activity of ceftazidime was increased at low concentrations 16 µg/ml and 32 µg/ml (Fig. 4). In contrast, the combination of tannase with ceftazidime has strong antibacterial activity against *S. aureus* since at the concentrations 16 µg/ml and 32 µg/ml, the diameter of inhibition zone reached to 26 mm and 28 mm, respectively, in comparison with ceftazidime alone (Fig. 4). On the other hand, the purified tannase without ceftazidime did not appear to show any antibacterial activity against the tested bacteria.

Fig. 3 Culture filtrate of *Penicillium expansum* grown in pomegranate pith medium

TABLE II
DIAMETER OF INHIBITION ZONES FOR *S. AUREUS* AND *PSEUDOMONAS AERUGINOSA* STRAINS IN PLATES WITH AND WITHOUT TANNASE

| Bacteria | Diameter of inhibition zone for cef. (mm) with different con. (µg/ml) | | | | Diameter of inhibition zone for cef. (mm) at different con. + tannase (1:1vol/vol) | | | | Diameter of inhibition zone for cef. (mm) at different con. + tannase (1:2 vol/vol) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. aureus* | 128 | 64 | 32 | 16 | 128 | 64 | 32 | 16 | 128 | 64 | 32 | 16 |
| | 28 | 25 | 23 | 20 | 28 | 28 | 24 | 24 | 28 | 28 | 26 | 28 |
| *P. aeruginosa* | 31 | 30 | 26 | 24 | 31 | 30 | 27 | 25 | 31 | 30 | 26 | 26 |




*Pseudomonas aeruginosa* *S. aureus*

Fig. 4 (a) Diameter of inhibition zones of purified tannase alone, (b) of ceftazidime alone, (c) of ceftazidime and tannase combination at 1:1 ratio, (d) of ceftazidime and tannase combination at 1:2 ratio against *Pseudomonas aeruginosa* and *S. aureus*

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